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Control of Transcription Termination by an RNA Factor in Bacteriophage P4 Immunity: Identification of the Target Sites

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Prophage P4 immunity is elicited by a short, 69-nucleotide RNA (CI RNA) coded for within the untranslated leader region of the same operon it controls. CI RNA causes termination of transcription that starts at the promoter P_{LE} and prevents the expression of the distal part of the operon that codes for P4 replication functions (α operon). In this work, we identify two sequences in the untranslated leader region of the α operon, *seqA* and *seqC*, that are the targets of the P4 immunity factor. *seqA* and *seqC* exhibit complementarity to a sequence internal to the CI RNA (*seqB*). Mutations in either *seqA* or *seqC* that alter its complementarity to *seqB* abolished or reduced P4 lysogenization proficiency and delayed the shutoff of the long transcripts originating from P_{LE} that cover the entire operon. Both *seqA* and *seqC* single mutants were still sensitive to P4 prophage immunity, whereas P4 *seqA seqC* double mutants showed a virulent phenotype. Thus, both functional sites are necessary to establish immunity upon infection, whereas a single site appears to be sufficient to prevent lytic gene expression when immunity is established. A mutation in *seqB* that restored complementarity to both *seqA* and *seqC* mutations also restored premature termination of P_{LE} transcripts, thus suggesting an important role for RNA-RNA interactions between *seqB* and *seqA* or *seqC* in P4 immunity.

In most known temperate bacteriophages, prophage immunity is determined by a repressor protein which prevents transcription initiation at promoters controlling the expression of replication functions and of other genes involved in the lytic cycle. Satellite bacteriophage P4 deviates from this model in two respects. (i) Prophage immunity does not prevent transcription initiation of the operon encoding the replication functions (α operon); rather, it promotes premature termination of transcription that initiates at P_{LE}, the constitutive promoter of the α operon (9, 10, 12). (ii) The immunity factor is not a protein but a small, 69-nucleotide (nt) RNA (CI RNA) exhibiting complementarity with the nontranslated leader region of the transcripts it regulates (10, 11a, 12).

P4 depends on all the morphogenetic genes of a helper phage such as P2 to assemble the viral particle. Autonomous P4 replication requires the expression of a primase-helicase coded for by the P4 α gene (14, 35) and leads either to the lytic cycle (when a helper phage genome is present in the host cell) or to a multicopy plasmid state (in the absence of the helper). As an episome, P4 may also integrate in the host chromosome and establish the lysogenic condition (for a review, see reference 21).

Central to the choice and the maintenance of the lysogenic versus the lytic or plasmid condition is the regulation of P4 replication genes (Fig. 1). Soon after infection of a sensitive host, transcription of the α operon starts at the constitutive promoter P_{LE}. Transcription from P_{LE} seems to be intrinsically termination prone, and transcripts of different lengths (0.3 to 0.5, 1.3, and 4.1 kb) are produced. At a later time as well as in the lysogenic state, production of transcripts longer than 0.3 to 0.4 kb initiating at P_{LE} is prevented by premature transcription termination (9, 10). This is dependent on a functional immunity RNA factor (CI RNA) which is coded for by the untranslated leader region of the α operon itself (*c*I gene) and is generated by the processing of the primary transcript (10, 11a, 12) (Fig. 1B). A region in the CI RNA (*seqB*) exhibits high complementarity with *seqA*, a sequence immediately downstream of P_{LE}. All P4 immunity-defective recessive mutants isolated so far carry a mutation in *c*I, and most of the mutations fall in *seqB*. This suggested that the P4 immunity factor controls termination of transcription that starts at P_{LE} by specific RNA-RNA interactions between CI and the nascent transcript (10, 12).

Thus, in P4, both immunity and replication functions are coded for by the same operon. Transcription from P_{LE} of the operon distal portion, which encodes the replication functions, occurs only soon after infection, before the CI RNA immunity factor is produced by the processing of the primary transcript. Later on, transcription from P_{LE} is restricted by enhancement of premature transcription termination efficiency to the proximal part of the operon which encodes the immunity factor itself.

Expression of the α operon late in the lytic cycle and in the plasmid condition is made possible by activation of P_{LL}, a positively regulated promoter located 400 nt upstream of P_{LE} (9). Transcription from P_{LL} is insensitive to transcription termination controlled by P4 immunity, probably because translation of two open reading frames (*orf88* and *orf199*) at the 5′ end of the P_{LL} mRNA protects the nascent transcript from the action of the immunity factor (Fig. 1) (23a).

To elucidate the peculiar immunity mechanism of P4 and understand how a small RNA may control transcription termination, it is fundamental to identify the target(s) of the RNA immunity factor. In this work, we show that the previously identified element *seqA* is indeed involved in the control of P4 immunity; moreover, we identify an additional site (*seqC*) in the untranslated leader region of the α operon which is also complementary to *seqB* and is involved in P4 lysogenization. Evidence that *seqA* and *seqC* represent the target sites for the CI RNA immunity factor is presented.

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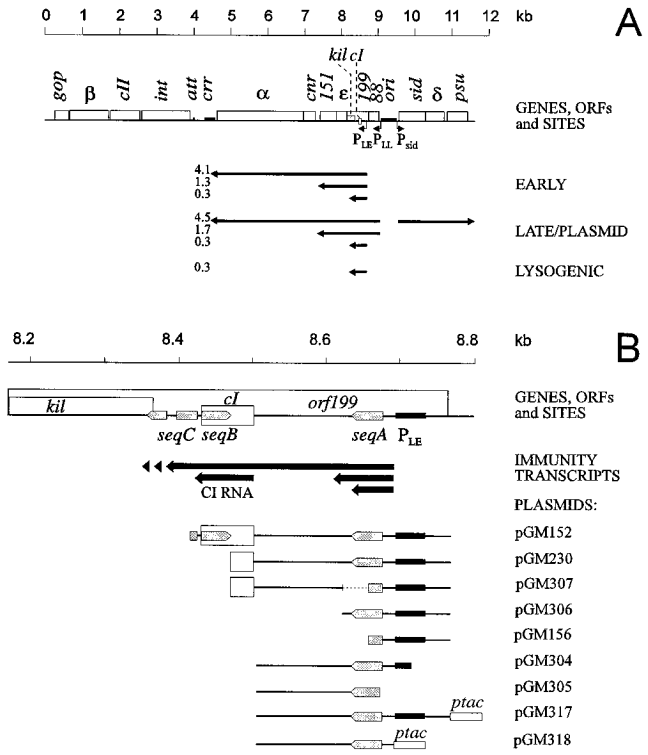

FIG. 1. P4 map. (A) Genetic and physical map of phage P4 and transcripts of P4 essential region. The transcripts produced during the early, late-plasmid, and lysogenic regulatory states are indicated by arrows; the transcripts of the lysogenic state are detailed in panel B. The sizes of the transcripts are indicated at the left in kilobases. (B) Map of the P4 immunity region and of cloned P4 DNA fragments. The fragments cloned in the expression vectors pTTQ18 and pTTQ19 are identified by the addition of the promoter (*ptac*). The *seqA* and *seqB* coordinates are 8676 to 8640 and 8479 to 8432, respectively; for those of *seqC*, see the text. The coordinates of the P4 DNA fragments are reported in Table 1. Data are from references 9, 10, 12, 15, 32, and this work. ORF, open reading frame.

MATERIALS AND METHODS

Bacteria, phages, and plasmids. The bacterial strains used were derivatives of *Escherichia coli* C-1a (prototrophic) (26), C-2404 [*galK*(Am) *recA*] (12), C-5201 (polyauxotrophic *str-1*) (7), C-5202 (polyauxotrophic *str-1 supD*) (7), and C-5505 (polyauxotrophic *str-1 pcnB*::Tn*10*) (12) and their P2 (P2 *lg*, a large plaque former) (2) and P4 lysogenic derivatives; in the text, the prophage is indicated in parentheses following the name of the parental strain. P4 derivatives and plasmids carrying P4 genome fragments are listed in Table 1. The plasmid vectors used were pKO1 (23), pTTQ18 and pTTQ19 (30), and pUC8 (33). The cloned P4 DNA fragments are diagrammed in Fig. 1. The P4 genome coordinates are from the updated P4 DNA sequence (GenBank accession number X51522) (15, 36).

pGM318 was constructed by cloning the P4 8496 to 8693 region in pTTQ18. The region was obtained by PCR of P4 DNA with the oligos A and B being used as primers (see below) and by digestion with *Alu*I. The *kpn1* mutation was created by overlap extension and PCR amplification (see below) of $P4^+$ 8594 to 9127 with the mutated primers kpn4 and npk5. The fragment, digested with *Mlu*I(8623) and *Xmn*I(9103), was cloned in pGM236 digested with the same enzymes, giving pGM324. *seqA405* and *seqA8* were created by overlap extension and PCR amplification of $P4^+$ 8594 to 8785 with the mutated primers 6-7 and 8-9, respectively. The fragments, digested with *Mlu*I (Klenow filled) and *Kpn*I, were cloned in pUC18 *Sal*I (Klenow filled)-*Kpn*I, giving pGM322 and pGM323, respectively. The *seqC405* mutation was created by overlap extension and PCR amplification of either $P4^+$ (to give pGM327) or the P4 c*I405* (pGM326) 8303 to 8645 fragment with the mutated primers 10 and 11, whereas to produce *seqC8* (pGM329), the oligos 12 and 13 were used. The fragments, digested with *Bgl*I(8371)-*Mlu*I(8624), were cloned in pGM13 treated with the same enzymes. The sequence of the cloned regions obtained by PCR was confirmed by sequencing.

All the site-specific mutations obtained by PCR mutagenesis were transferred in the phage genome by in vitro recombination of suitable restriction fragments, and the mutant phage was recovered by transfection of C-5202(P2) as described (1). The genotype of the recombinant phage was confirmed by sequencing the relevant region.

Oligonucleotides and RNA probes. The oligonucleotides used for PCR are the following (the coordinates are those of the P4 sequence in the oligo 5′ to 3′ direction, and the base changes from the P4 sequence [P4 base-position-base change] are reported): oligo 6, 8679 to 8645, G8663A; oligo 7, 8647 to 8680, C8663T; oligo 8, 8679 to 8645, A8662G; oligo 9, 8647 to 8680, T8662C; oligo 10, 8393 to 8426, C8411T; oligo 11, 8426 to 8394, G8411A; oligo 12, 8393 to 8426, T8410C; oligo 13, 8426 to 8394, A8410G; oligo A, 8361 to 8380; oligo B, 8693 to 8680; oligo kpn4, 8793 to 8759, G8772C; oligo npk5, 8749 to 8783, C8772G; oligo vir^+, 9088 to 9110; and oligo vir1, 9088 to 9110, G9099A. The γ-^{32}P-labelled RNA probes P_{LE}-t2 and SeqB were prepared as described (10) and cover P4 8418 to 8774 and 8418 to 8470, respectively.

P4 mutagenesis and screening of P4 virulent mutants by lysis spot hybridization. UV light mutagenesis of P4 was performed as described by Ghisotti et al. (12). To identify virulent mutants, the P4 clear plaques obtained were purified and replica plated on C-5201(P2)(P4). Spontaneous virulent mutants were obtained by plating about 10^8 P4 PFUs on the P2-P4 double lysogen.

To distinguish between *vir1*-like (promoter up mutations in P_{LL}) and virulent mutants of a different kind, spontaneous and UV-induced virulent phages were hybridized to ^{32}P-labelled vir1 or vir^+ oligonucleotides corresponding to either the P4 *vir1* or the $P4^+$ −10 region of the P_{LL} promoter, respectively. P4 virulent plaques were resuspended in microtiter wells and replica plated on C-5201(P2 *lg*) indicator. The P4 DNA of the lysis spots was immobilized on two nitrocellulose filters as described previously for phage lambda plaques (25), and the filters were hybridized to vir1 and vir^+ oligonucleotides that were 5′ end labelled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP, as described previously (25). Hybridization was performed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7) containing 0.05% sodium pyrophosphate and 0.5% sodium dodecyl sulfate at 48°C for 18 h. The filters were washed three times at room temperature and once at 58°C with 6× SSC–0.05% sodium pyrophosphate. These conditions allowed specific hybridization only to the fully complementary DNA, whereas a single mismatch was sufficient to prevent hybridization.

Base-directed mutagenesis by PCR overlap extension. Specific base substitution mutants were generated by the procedure described by Ho et al. (17). In summary, two complementary synthetic oligonucleotides carrying the desired base substitution and two external primers were used in two independent PCRs to generate two DNA fragments having one overlapping end sequence. These two fragments were combined in a subsequent PCR in which the annealed overlapping ends of each strand served as primers for the 3′ extension on the complementary strand, thus generating a fusion DNA molecule further amplified by the external primers. The fragment so obtained was cut by restriction enzymes and cloned in a suitable plasmid. Both strands of the region containing the mutation to be used for the construction of other recombinant plasmids and phage were fully sequenced. In order to limit the region to be sequenced and to facilitate the transfer of the desired mutation in P4, we created the *kpn1* mutation in pGM324 by PCR mutagenesis and transferred it in P4. P4 *kpn1*, which carries a unique *Kpn*I restriction site, was then used for all subsequent P4 mutant strain constructions.

Complementation test of P4 clear plaque mutants. Ten microliters (each) of two clear plaque mutant stocks of about 10^8 PFU ml^{-1} were cross streaked on C-5201(P2 *lg*) indicator. Turbid indicator outgrowth at the crossing of the two clear streaks indicated complementation for immunity.

P4 one-step growth and screening for P4 lysogens in a P2 lysogenic host. The bacterial strains were grown at 37°C in LD broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented with 5 mM $CaCl_2$ (to allow P4 adsorption) and 100 μg of ampicillin ml^{-1} (if the strain carried an Amp^r plasmid) to 40 Klett colorimeter units, equivalent to 10^8 cells ml^{-1}. When induction of the P_{tac} promoter of the resident plasmid was required, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 40 μg ml^{-1} and the cultures were further incubated for 1 h at 37°C and diluted to 40 Klett units before the infection. The cells were then infected with P4 with the intent of achieving a multiplicity of infection of 10 and incubated at 37°C. After 15 min, phage adsorption was measured and P4 antiserum (K value, 1) was added to inactivate the unadsorbed phage; after 20 min, the cells surviving the infection and the cells yielding phage progeny (yielders) were assayed. A culture sample was diluted 10^4-fold in LD (with ampicillin if the strain carried an Amp^r plasmid) and incubated for 40 min at 37°C with aeration, and the phage titer produced was assayed. The clones surviving the infection were analyzed for lysogeny by testing their ability to release the prophage and their sensitivity to P4 c*I405* and P4 *vir1*.

Infection with P4 and screening for P4 lysogens and plasmid carriers in the absence of P2 (*cox* test). Cell growth, infection with P4, and measurement of the survivors were performed as in the one-step-growth experiment. The screening of the lysogens and P4 plasmid carriers among the survivors was performed as described previously (8). The test is based on the fact that the helper P2 *cox* gene is necessary to induce the P2 lytic cycle upon P2 infection of a P4 lysogen but is not required when P4 is in the plasmid condition (8, 29). Thus, the P4 lysogens were identified as the clones that released P4 following infection with P2 cox^+ but not with P2 *cox4*, whereas P4 plasmid carriers among the survivors released P4 following infection with both P2 cox^+ and P2 *cox4*.

Northern (RNA) blot hybridization. RNA was extracted from *E. coli* and from P4-infected cells as described previously (9) and fractionated by either 1.5%

TABLE 1. Bacteriophage P4 derivatives and plasmids

Bacteriophage or plasmid (vector)	Relevant properties and origin (bacteriophages) or cloned P4 region (plasmids)	Mutation	Source or reference
Bacteriophages			
P4	Wild type		28
P4 *ash3*	C8477A mutation in *c*I		20
P4 *ash8*	T8447C mutation in *c*I		20
P4 *ash9*	T8448A mutation in *c*I		20
P4 *ash29*	G8433A mutation in *c*I		20
P4 *ash29 seqC30*	Spontaneous virulent mutant from P4 *ash29*; C8382A mutation in *seqC*		This work
P4 *ash29 seqC31*	Spontaneous virulent mutant from P4 *ash29*; C8382A mutation in *seqC*		This work
P4 *cI9*	C8446T mutation in *c*I (same as *cI405*)		4, this work
P4 *cI10*	T8473C and C8474G double mutation in *c*I		4, this work
P4 *cI405*	C8446T mutation in *c*I		4, 20
P4 *cIIam3*	Makes clear plaques, complements *c*I mutants		4
P4 *kpn1*	G8772C mutation, creates a unique *Kpn*I restriction site; by substitution of P4 *Mlu*I(8623)-*Apa*LI(10651) fragment of pGM324 in $P4^+$		This work
P4 *kpn1 cI405*	By substitution of P4 *Mlu*I(8623)-*Apa*LI(10651) fragment of pGM324 in P4 *cI405*		This work
P4 *kpn1 seqA8*	A8662G mutation in *seqA*; by substitution of P4 *Mlu*I(8623)-*Kpn*I(8772) fragment of pGM323 in P4 *kpn1*		This work
P4 *kpn1 seqA8 seqC8*	By substitution of P4 *Mlu*I(8623)-*Kpn*I(8772) fragment of pGM323 in P4 *kpn1 seqC8*		This work
P4 *kpn1 seqA405*	G8663A mutation in *seqA*; by substitution of P4 *Mlu*I(8623)-*Kpn*I(8772) fragment of pGM322 in P4 *kpn1*		This work
P4 *kpn1 seqA405 cI405 seqC32*	Spontaneous virulent mutants from P4 *kpn1 seqA405 cI405*; *seqC32* is G8419T		This work
P4 *kpn1 seqA405 cI405 seqC33*	Spontaneous virulent mutant from P4 *kpn1 seqA405 cI405*; *seqC33* is T8407C		This work
P4 *kpn1 seqA405 cI405 seqC405*	By substitution of P4 *Mlu*I(8623)-*Kpn*I(8772) fragment of pGM326 in P4 *kpn1 seqA405*		This work
P4 *kpn1 seqA405 seqC405*	By substitution of P4 *Tth*111I(7772)-*Mlu*I(8623) fragment of pGM327 in P4 *kpn1 seqA405*		This work
P4 *kpn1 seqC8*	A8410G mutation in *seqC*; by substitution of P4 *Tth*111I(7772)-*Mlu*I(8623) fragment of pGM329 in P4 *kpn1*		This work
P4 *kpn1 seqC405*	G8411A mutation in *seqC*; by substitution of P4 *Tth*111I(7772)-*Mlu*I(8623) fragment of pGM327 in P4 *kpn1*		This work
P4 *seqC30*	By ligation of fragments *Taq*I(8418)-*Mlu*I(8623) and *Mlu*I(8623)-*cos*-*Tth*111I(7772) of $P4^+$ with fragment *Tth*111I(7772)-*Taq*I(8418) of P4 *ash29 seqC30*		This work
P4 *vir1*	C9099T mutation in P_{LL}		22
Plasmids (vectors)			
pGM13 (pUC8)	*Dra*I(7656)-*Dra*I(9191)		This work
pGM85 (pKO1)	*Taq*I(8418)-*Rsa*I(8774)	*cI405* (C8446T)	This work
pGM152 (pKO1)	*Taq*I(8418)-*Rsa*I(8774)		12
pGM156 (pKO1)	*Ava*I(8656)-*Rsa*I(8774)		12
pGM230 (pKO1)	*Hae*III(8471)-*Rsa*I(8774)		12
pGM236 (pUC8)	*Hin*cII(6447)-*Bam*HI(10657)		This work
pGM304 (pKO1)	*Alu*I(8496)-*Hpa*II(8719)		This work
pGM305 (pKO1)	*Alu*I(8496)-*Hpa*II(8674)		This work
pGM306 (pKO1)	*Mlu*I(8623)-*Rsa*I(8774)		12
pGM307 (pKO1)	*Hae*III(8471)-*Rsa*I(8774)	Deletion *Mlu*I(8623)-*Ava*I(8656)	This work
pGM317 (pTTQ19)	*Alu*I(8496)-*Rsa*I(8774)		This work
pGM318 (pTTQ18)	*Alu*I(8496)-8693		This work
pGM322 (pUC18)	*Mlu*I(8623)-*Kpn*I(8772)	*seqA405* (G8663A)	This work
pGM323 (pUC18)	*Mlu*I(8623)-*Kpn*I(8772)	*seqA8* (A8662G)	This work
pGM324 (pUC18)	*Hin*cII(6447)-*Bam*HI(10657)	*kpn1* (G8772C)	This work
pGM326 (pUC8)	*Dra*I(7656)-*Dra*I(9191)	*cI405 seqC405*	This work
pGM327 (pUC8)	*Dra*I(7656)-*Dra*I(9191)	*seqC405* (G8411A)	This work
pGM329 (pUC8)	*Dra*I(7656)-*Dra*I(9191)	*seqC8* (A8410G)	This work

formamide-formaldehyde agarose or 10% polyacrylamide-urea denaturing gel electrophoresis, following the procedure detailed by Sambrook et al. (25). RNA was transferred from the agarose gels onto Hybond-N filter membranes (Amersham) by vacuum blotting and from the acrylamide gels onto the same type of membranes by electroblotting. Hybridization to the specific riboprobes was performed as described previously (10).

RESULTS

Transcription of cloned *seqA* interferes with the establishment of P4 immunity. In a previous study, it was shown that a cloned 357-bp fragment of P4 DNA encompassing P_{LE}, *seqA*,

TABLE 2. Interference with P4 lysogenization by cloned fragments of the P4 immunity region

Plasmid carried	Host infected: C-2404(P2)				Host infected: C-2404			
	Survivors (%)	Yielders (%)[a]	Lysogens (%)[b]	Lysogenization index[c]	Survivors (%)	P4 plasmid carriers (%)[b]	Lysogens (%)[b]	Lysogenization index[c]
pKO1	38.0	81	98	4.6×10^{-1}	140	0.33	88	267
pGM230	1.1	104	89	9.4×10^{-3}	83	11.0	86	7.8
pGM306	0.4	125	78	2.4×10^{-3}	63	47.0	49[d]	1.0
pGM156	29.0	88	100	3.3×10^{-1}	86	<0.6	93	>155
pGM307	27.0	89	ND[e]		122	0.3	82	273
pGM304	24.0	64	ND		64	1.9	88	46.3
pGM305	31.0	96	ND		114	0.3	81	270
pTTQ	25.5	62	66	2.7×10^{-1}				
pTTQ[f]	30.3	47	82	5.2×10^{-1}				
pGM317	1.2	149	80	6.4×10^{-3}				
pGM317[f]	0.8	147	80	4.3×10^{-3}				
pGM318	36.7	50	89	6.7×10^{-1}				
pGM318[f]	0.3	90	61	1.9×10^{-3}				

[a] Relative to the infected cells; yielders were assayed 20 min after infection, which may justify the greater than 100% figures.
[b] Among survivors.
[c] Ratio of lysogens to either plasmid carriers (in C-2404) or yielders (in the P2 lysogen) normalized to the infected cells.
[d] Lysogens were unstable and segregated P4 plasmid carriers.
[e] ND, not determined.
[f] In these cases, 40 μg of IPTG ml^{-1} was added both to the culture 60 min before P4 infection and to the plates for the assay of survivors.

and *c*I (pGM152 on Fig. 1B) conferred P4 immunity to the bacterial host, thus preventing P4 plaque formation, whereas shorter fragments (pGM230 and pGM306) containing P_{LE} and *seqA* caused P4 to make clear plaques. An even shorter fragment (pGM156) missing the distal part of *seqA* did not affect the P4 turbid plaque morphology. This suggested that the presence of *seqA* without *c*I on the plasmid vector interfered with P4 lysogenization by titrating out the immunity factor produced by the superinfecting phage and that interactions between the complementary sequences *seqA* and *seqB* might be involved in P4 immunity control (10, 12).

To define whether the cloned fragments discussed above would actually interfere with P4 immunity establishment and/or maintenance, we tested their effect on the choice of the lysogenic versus the lytic or plasmid condition. Cells harboring the appropriate plasmid were infected with P4; 20 min later, we assayed the cells surviving the infection and, in the P2 lysogen, the cells producing P4 phage (yielders). The survivors were then tested for P4 lysogeny and, in the P2 nonlysogenic host, for the presence of P4 in the plasmid condition, as described in Materials and Methods. The lysogenization index was calculated as the ratio of the lysogenic cells to either the yielders or the P4 plasmid carriers. To avoid recombination between the infecting phage and the resident plasmid, the experiments were performed in *recA* hosts.

In the P2 lysogenic host (Table 2), the cloned P4 fragments containing both P_{LE} and *seqA* (pGM230 and pGM306) caused a reduction of the cell fraction surviving P4 infection and an increase in yielder frequency by comparison with the controls (the vector pKO1 and pGM156 that carries P_{LE} and only the 5′ half of *seqA*). In the absence of P2 (Table 2), pGM230 and pGM306 greatly increased the frequency of clones carrying P4 as a plasmid. Nevertheless, P4 lysogens could be recovered both in the presence and in the absence of P2. Thus, it appeared that the P4 cloned fragments would favor the lytic cycle and the establishment of the plasmid state and greatly reduce the lysogenization index. We shall call this effect anti-immunity.

To define more precisely the elements required for anti-immunity, we made deletions of either *seqA* (pGM307) or P_{LE} (pGM304 and pGM305) (the former is missing the −35 region of P_{LE}, whereas in the latter, the deletion completely removes the promoter and the first 4 nt of *seqA* [Fig. 1]). Deletion of either *seqA* or P_{LE} was sufficient to abolish the anti-immunity effect (see data for pGM307, pGM304 and pGM305 [Table 2]). Moreover, a plasmid carrying the *seqA* region under the control of the inducible promoter P_{tac} (pGM318) exhibited the anti-immunity effect only when transcription was induced by IPTG (Table 2), suggesting that transcription of the *seqA* region was necessary for the anti-immunity effect to occur. IPTG did not affect the outcome of P4 infection in the control plasmid pGM317 in which *seqA* can be transcribed both from P_{tac} and the constitutive promoter P_{LE}.

The P4 lysogens of the strains carrying the anti-immunity plasmids could be stably maintained. In the presence of P2, the titer of P4 spontaneously released by the strains carrying the anti-immunity fragments was 10- to 100-fold higher than that of control strains; in the absence of P2, segregation of P4 plasmid carriers was detectable in the strain carrying pGM306 (up to 50% of P4 plasmid-carrying clones in overnight cultures) but was below the detection limit ($<10^{-3}$) with pGM230.

These data indicate that transcription of the P4 *seqA* region from a plasmid vector interferes with the establishment of P4 immunity but that it is compatible with the maintenance of the lysogenic state.

Transcription of the P4 α operon from P_{LE} in the presence of anti-immunity plasmids. We then tested whether the anti-immunity plasmids would interfere with the normal P4 transcription pattern from P_{LE}. Cells carrying either the anti-immunity plasmid pGM230 or the control vector pKO1 were infected with P4; the RNA was extracted at different times after infection and analyzed by Northern blot hybridization with a P_{LE} proximal probe. As can be seen from Fig. 2A, the early 4.1-kb mRNA, which covers the entire α operon, was no longer detectable 20 min after infection of control cells, as previously observed (9, 10). In contrast, in cells carrying the anti-immunity plasmid pGM230, the amount of the 4.1-kb mRNA greatly increased up to 20 min after P4 infection (expression of the 1.3-kb mRNA produced by P4 could not be analyzed in this experiment because of the presence of transcripts of a similar size produced by pGM230). This RNA

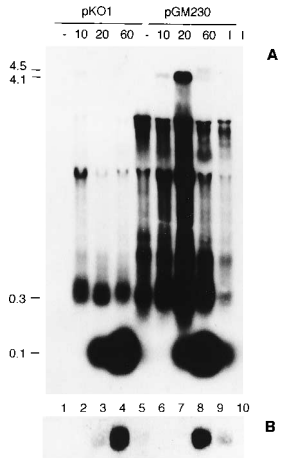

FIG. 2. Northern blot analysis of P4 α operon transcription in the presence of the anti-immunity plasmid pGM230. RNA was extracted from cultures of C-2404(pKO1) (control vector plasmid) or C-2404(pGM230) (which carries the anti-immunity region) before infection (−) and 10, 20, and 60 min following P4 infection, as indicated at the top of panel A (lanes 1 to 8); lanes 9 and 10 are noninfected P4 lysogenic (l) strains C-2404(P4)(pGM230) and C-2404(P4), respectively. (A) The RNA was fractionated by 1.5% agarose gel electrophoresis and hybridized to the riboprobe P_{LE}-t2 (see Materials and Methods). The apparent sizes of the $P4^+$ transcripts, as previously determined (9, 10), are indicated in kilobases on the left. Hybridization signals longer than 0.3 kb in the P4 lysogenic C-2404(pGM230) host are of pGM230 origin, as evidenced by the lack of hybridization with a P4 P_{LE} distal probe (data not shown). (B) The RNA (lanes 1 to 9) was fractionated by 10% acrylamide–7 M urea gel electrophoresis and hybridized to the riboprobe SeqB (see Materials and Methods). Only the bands corresponding to CI RNA are shown.

species, however, almost disappeared 60 min after infection and could not be detected in the P4 lysogen. We also used Northern blot hybridization with a CI RNA specific probe to examine whether the anti-immunity plasmid would interfere with the production of the P4 immunity factor. As can be seen from Fig. 2B, no substantial difference between the anti-immune host and the control was apparent with respect to the time and the amount of CI RNA production.

It thus appears that the presence of the anti-immunity plasmid greatly delays the shutoff of long mRNAs originating at P_{LE} upon P4 infection. However, the anti-immunity plasmid did not interfere significantly either with the synthesis of the CI RNA or with the P4 transcription pattern of a lysogenic cell.

Effect of *seqA* mutations on anti-immunity. The simplest explanation for the anti-immune effect is that the RNA produced by the cloned fragment titrates out the CI RNA produced by the infecting phage; this would delay the turnoff of long-mRNA production, thus opposing the establishment of P4 immunity.

Titration of the P4 immunity factor could depend on RNA-RNA interactions between the complementary sequences *seqA* and *seqB*. To test this idea, we introduced two mutations in the anti-immunity fragment of pGM306 by in vitro site-specific mutagenesis (17) and tested their effect on anti-immunity. These mutations altered the complementarity of *seqA* with *seqB* (*seqA8* [A8662G] and *seqA405* [G8663A]) (Fig. 3). For reasons that will become apparent, the base substitutions *seqA8* and *seqA405* were such as to restore complementarity with the mutated *seqB* of the known P4 immunity-defective mutants *ash8* and *cI405*, respectively. The *seqA405* mutation in pGM322 abolished anti-immunity, as evidenced by the turbid plaques made by P4 on a strain carrying this plasmid, thus supporting the idea of a *seqA-seqB* interaction via complementary base pairing. The *seqA8* mutant plasmid (pGM323), however, still caused P4 to form clear plaques and thus exhibited the anti-immunity effect. This might be due to the fact that the A-to-G transition in *seqA8* may still allow pairing with the corresponding U in *seqB*.

***seqA* is necessary for P4 immunity.** The data given above support the hypothesis that *seqA* is an RNA target for the P4 CI immunity factor. However, they do not define the role that *seqA* may play in the bacteriophage immunity mechanism, in which *seqA* might function either as an anti-immunity determinant or as an element necessary for immunity. To investigate this further, we transferred the *seqA* mutations into P4 by in vitro recombination. Both P4 *seqA8* and P4 *seqA405* made clear plaques and were unable to lysogenize in both P2 lysogenic and nonlysogenic strains (Table 3); however, contrary to the expectation for mutants in the target of the immunity factor, they did not form plaques on P4 lysogenic hosts and were therefore sensitive to P4 immunity (Table 4 and data not shown). This suggests either that *seqA* is not a target for the immunity factor or that an additional target(s) is involved in the P4 immunity mechanism and that multiple mutations are required for virulence.

In a cross-streak complementation test with P4 clear plaque mutants (see Materials and Methods), P4 *seqA8* and *seqA405* mutants did not complement one another or *c*I mutants for immunity (the streaks remained clear at the crossing with P4 *cI405*, P4 *ash3*, and P4 *ash29*), whereas complementation was observed (turbid area at the crossing) with P4 *c*II*am3*, a clear plaque mutant that belongs to the *c*II complementation group (4, 13). Thus, both P4 *seqA8* and *seqA405* appear to be recessive immunity-sensitive mutants.

Isolation of P4 mutants insensitive to immunity: identification of the *seqC* locus. In order to identify the target(s) for the P4 immunity factor, we sought mutants that were insensitive to immunity. The only virulent mutant characterized so far, P4 *vir1*, carries a promoter up mutation in the −10 region of P_{LL} and activates transcription from this promoter, which is insensitive to transcription termination controlled by P4 immunity (9, 20, 22).

To identify virulent mutants of a different kind, we screened P4 virulent mutants by plaque hybridization with ^{32}P-labelled oligonucleotides corresponding either to the wild type or to the *vir1* −10 region of P_{LL} (see Materials and Methods). In a first survey of spontaneous and UV-induced P4 virulent mutants derived either from a wild-type phage or from immunity-defective *c*I mutants (*ash8*, *ash9*, *cI9*, *cI10*, and *cI405*) (Table 1), 14 out of 14 independent mutants analyzed carried a mutation in P_{LL}. It thus appears that when P4 virulent mutants are selected for, P_{LL} promoter up mutations are most likely obtained.

To circumvent this problem, we selected for virulent derivatives of P4 *ash29* and P4 *seqA405 cI405* mutants. *ash29* (G8433A) is a pleiotropic mutation that confers a weak immunity defect on P4 and suppresses the virulence of P4 *vir1* (19, 27a). This latter phenotype appears to be due to the polarity of the amber mutation created in *orf199* that leads to the premature termination of transcription starting at P_{LL} (23a). The suppression of virulence could therefore decrease the incidence of P_{LL} promoter up mutations. On the other hand, if multiple mutations were required for virulence, the presence of *seqA405* could increase the frequency of virulent mutants.

From P4 *ash29*, we obtained and analyzed two independent virulent derivatives that hybridized only to the oligo vir^+ and carried an additional mutation (*seqC30* and *seqC31*, respectively); both mutations turned out to be the same C8382A transversion. From P4 *seqA405 cI405*, we isolated the virulent derivatives *seqC32* (G8419T) and *seqC33* (T8407C). These

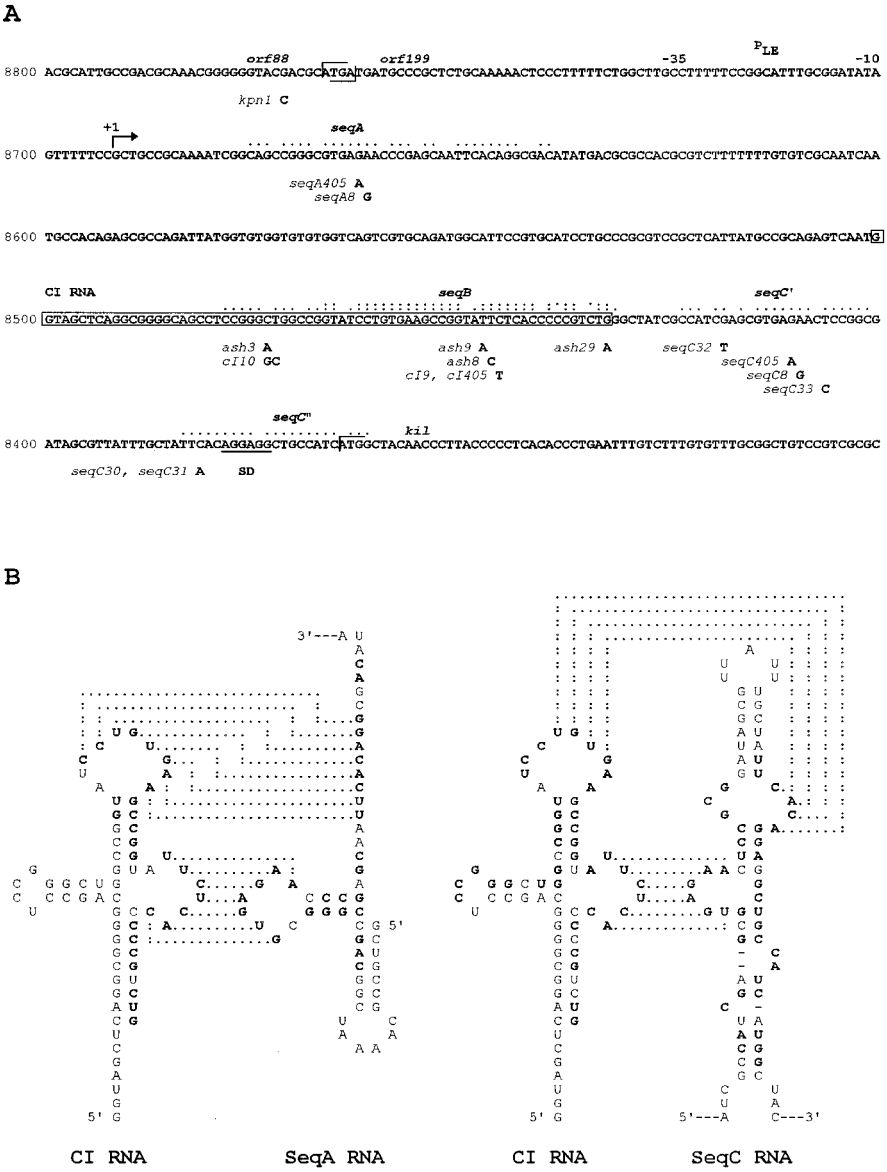

FIG. 3. Immunity region of bacteriophage P4 and interactions between CI RNA and *seqA-seqC*. (A) The complementary strand of the P4 8300 to 8800 region (15) is shown. Under the sequence are indicated the mutations in this region that are mentioned in the text. *seqA* and *seqC* are indicated by dots. The region coding for CI RNA is boxed; the bases in the *seqB* region complementary to *seqA* and to *seqC* are indicated by the upper and the lower set of dots, respectively. ⌜, transcription startpoint (+1) of P_{LE}; ⌟, stop codon of *orf88*; ⌜, start codons of *orf199* and *kil*. The ribosome-binding site for *kil* (SD) is underlined. (B) Predicted secondary structure of the CI RNA and possible interactions with the 5′ end of the P_{LE} transcript (SeqA RNA) and the region containing *seqC* (SeqC RNA). *seqB-seqA* and *seqB-seqC* complementary bases are in boldface type. The complementary bases in single-stranded regions are connected by dotted lines. Data are from references 9, 10, 11a, 12, 15, and 21 and this work.

mutations drew our attention to a region we called *seqC*, which, like *seqA*, presents high complementarity with *seqB*. *seqC* is located downstream of *c*I and is split into two portions, *seqC′* (8402 to 8424) and *seqC″* (8362 to 8384), by a 17-nt sequence with hyphenated dyad symmetry; all of the spontaneous mutations in *seqC* listed above are changes in bases complementary to *seqB* (Fig. 1 and 3).

The isolation of such mutants indicates that *seqC* is involved in the control of P4 immunity. To study its role, we constructed a P4 *seqC30* single mutant by in vitro recombination; moreover, we created two additional mutations in *seqC* by base-specific mutagenesis. These mutations, *seqC8* (A8410G) and *seqC405* (G8411A) (see Materials and Methods) (Fig. 3), altered the complementarity with *seqB*; such mutations would also restore the complementarity of *seqC* with *seqB* for *ash8* and *cI405* mutants, respectively. Then, by in vitro recombination, we constructed a number of P4 mutants carrying single or multiple mutations in *seqA*, *seqB*, and *seqC* (Table 1) and tested their effect on P4 lysogenization (Table 3).

In a P2 lysogenic strain, both P4 *seqC30* and P4 *seqC405* single mutants gave clear plaques and were defective in lysogenization. In the absence of the helper, both P4 lysogens and plasmid-carrying clones could be obtained, although both types appeared to be unstable (Table 3). However, like the *seqA* mutants, the *seqC* single mutants could not form plaques on a P2-P4 double lysogen (Table 4 and data not shown); thus, they were still sensitive to P4 prophage immunity. By a cross-streak complementation test, they appeared to belong to the *c*I

TABLE 3. Effect of *seqA* and *seqC* mutations on P4 lysogenization

Infecting phage[a]	Host infected: C-1a(P2 *lg*)					Host infected: C-1a			
	Survivors (%)	Yielders (%)	Burst size[b]	Lysogens (%)[c]	Lysogenization index[d]	Survivors (%)	P4 plasmid carriers (%)[c]	Lysogens (%)[c]	Lysogenization index[d]
P4	61	12.5	14	100	4.9	100	1.1	66	60
P4 *cI405*	0.17	131	53	<2	$<2.5 \times 10^{-5}$	0.2	4	<2	<0.5
P4 *seqA8*	0.27	93	114	<2	$<5.8 \times 10^{-5}$	68	3.5[e]	<1.2	<0.35
P4 *seqA405*	0.29	101	68	<2	$<5.7 \times 10^{-5}$	45	10	<1.3	<0.13
P4 *seqC8*	60	47	75	96	1.2	88	2.1	85	40.5
P4 *seqC30*	6.1	90	91	85	5.8×10^{-2}	72	77[f]	12[g]	0.16
P4 *seqC405*	3.5	108	102	89	2.9×10^{-2}	76	90[f]	8.8[g]	0.1
P4 *seqA405 seqC405*	0.36	123	71	<2	$<5.8 \times 10^{-5}$	1.1	1.1[e]	<1.1	<1
P4 *seqA405 cI405 seqC405*	0.07	107	80	<2	$<1.3 \times 10^{-5}$	27	98	<1.1	<0.01

[a] All phages but P4 *seqC30* carried the additional *kpn1* mutation.
[b] PFUs produced per yielder.
[c] Among survivors.
[d] Ratio of lysogens to either plasmid carriers (in C-1a) or yielders (in the P2 lysogen) among infected cells.
[e] These plasmid carriers were unstable and gave cured clones upon streaking.
[f] P4 induction by P2 was *cox* independent; however, these clones were rather unstable and segregated *cox*-dependent clones upon streaking (see Materials and Methods).
[g] P4 induction by P2 was *cox* dependent; however, these clones were rather unstable and segregated *cox*-independent clones upon streaking.

complementation group. In contrast, P4 *seqA405 seqC405* was able to grow on a P4 lysogen as well as on a strain expressing P4 immunity from a multicopy plasmid (pGM152); thus, the double mutant exhibited a virulent phenotype (Table 4).

P4 *seqC8* formed turbid plaques and did not seem to be affected in lysogenization (Table 3); the double mutant P4 *seqA8 seqC8* gave clear plaques like those of the single *seqA8* mutant and was still sensitive to wild-type P4 immunity (data not shown).

Effect of a compensatory mutation in *seqB* on P4 *seqA405 seqC405* virulence. The data given above indicate that both *seqA* and *seqC* regions must be mutated in order to make a P4 phage insensitive to the prophage immunity. This suggests that both *seqA* and *seqC* represent functional targets for the P4 CI immunity factor. If CI RNA-target interactions depend on the complementarity of *seqB* with both *seqA* and *seqC*, a compensatory mutation in *seqB* that restored the complementarity between the CI RNA and the mutant target sites should also restore immunity. To test this prediction, we constructed the triple mutant P4 *seqA405 cI405 seqC405* and assayed its ability to lysogenize a sensitive host. As can be seen from Table 3 and 4, the *cI405* compensatory mutation in the phage was not sufficient to restore the ability to form turbid plaques and to lysogenize. However, neither this triple mutant nor the virulent P4 *seqA405 seqC405* double mutant could grow lytically when a CI RNA carrying the *cI405* mutation was expressed from a multicopy plasmid (pGM85), whereas they were insensitive to the wild-type immunity expressed by pGM152. Surprisingly, $P4^+$ could not grow with either pGM152 or with pGM85 (Table 4) (see Discussion).

TABLE 4. Efficiency of plating and plaque morphology of P4 mutants in the presence of wild-type or mutant P4 immunity factor

P4 genotype[a]			Efficiency of plating and plaque morphology[b]			
seqA	*seqB*	*seqC*	None	(P4)	pGM152[c]	pGM85[d]
+	+	+	1 (t)	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
+	405	+	1 (c)	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
405	+	+	1 (c)	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
+	+	405	1 (c)	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
405	+	405	1 (c)	1 (c)	1 (c)	$<10^{-4}$
405	405	405	1 (c)	1 (c)	1 (c)	$<10^{-4}$

[a] +, wild type; 405, mutant.
[b] Plating was done on C-5201(P2)[−] on the same strain lysogenic for P4 [(P4)] or carrying the plasmids pGM152 or pGM85. t, turbid; c, clear.
[c] Expresses the wild-type CI RNA.
[d] Expresses the CI405 mutant RNA.

Transcription pattern of P4 *seqA* and *seqC* mutants. The effect of the P4 mutations discussed above on transcription from P_{LE} was then tested by Northern blot analysis. The results are shown in Fig. 4. Unexpectedly, the *kpn1* mutation, which was introduced into P4 in order to facilitate the in vitro construction of P4 mutant strains, altered the wild-type transcription pattern even though it was located upstream of P_{LE} and did not affect P4 lysogenization efficiency. In P4 *kpn1*, the synthesis of the 4.1- and 1.3-kb transcripts was delayed and the peak of the production of these mRNAs occurred 20 min postinfection (in the P4 wild type, the peak is at 10 min) (9, 10). Nevertheless, at 60 min postinfection, the 4.1- and 1.3-kb

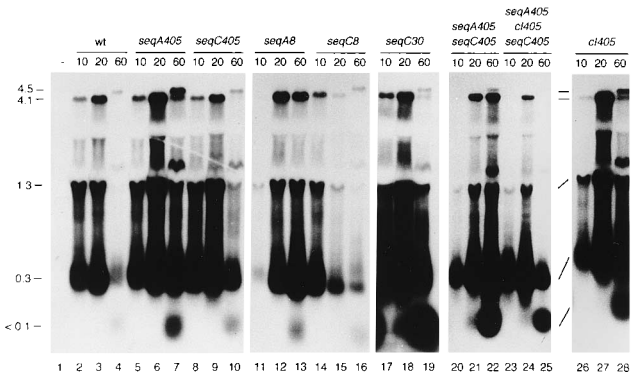

FIG. 4. Northern blot analysis of α operon transcription in P4 *seqA*, *seqB*, and *seqC* mutants. RNA extracted from strain C-1a infected with P4 was fractionated by 1.5% agarose gel electrophoresis and analyzed by Northern blot hybridization with the riboprobe P_{LE}-t2. The infecting phage mutants and the times after P4 infection of the RNA extractions (in minutes) are indicated at the top of the figure (−, noninfected cells). The sizes of the $P4^+$ transcripts (10) are indicated in kilobases at the left. All the phages used carried the additional *kpn1* mutation.

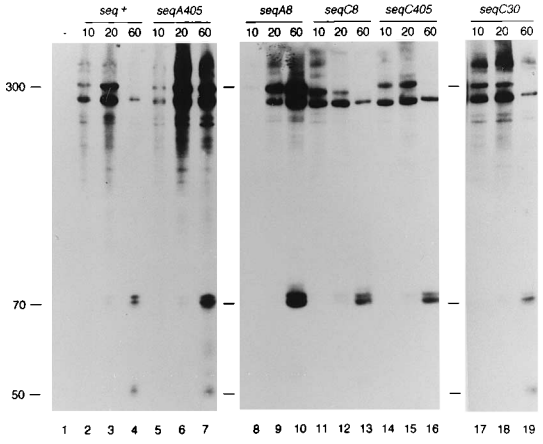

FIG. 5. Northern blot analysis of the transcripts from the immunity region of P4 *seqA* and *seqC* mutants. The RNA was fractionated by 10% acrylamide–7 M urea gel electrophoresis, electroblotted, and hybridized to probe P_{LE}-t2. The sizes of the transcripts are indicated in kilobases at the left. The times after infection of the extractions (in minutes) are indicated at the top of the figure (−, noninfected cells). All the phage used carried the additional *kpn1* mutation.

mRNAs disappeared and the 4.5- and 1.7-kb transcripts from P_{LL} were produced.

For all the *seqA* and *seqC* single mutants that were affected in lysogenization (thus excepting *seqC8*), the turnoff of the 4.1- and 1.3-kb RNAs was further delayed relative to that for the P4 *kpn1* control. In such mutants, a substantial amount of the 4.1- and 1.3-kb transcripts was still present 60 min after infection, and this was larger in *seqA* than in *seqC* mutants (Fig. 4). None of the tested *seqA* or *seqC* single mutants produced detectable amounts of RNA longer than 300 to 400 nt upon infection of a P4 lysogen (data not shown).

The virulent mutants P4 *ash29 seqC30* and P4 *seqA405 seqC405* did not turn off expression of the 4.1- and 1.3-kb transcripts even 60 min postinfection and produced this mRNA species even in the presence of P4 immunity (Fig. 4 and data not shown). Interestingly, the triple mutant P4 *seqA405 cI405 seqC405*, although unable to establish immunity, turned off the late expression of the 4.1- and 1.3-kb mRNAs. Thus, restoration of complementarity between *seqB* and *seqA* or *seqC* suppressed the transcription termination defect observed in the single and double mutants.

The short transcripts from the immunity region were analyzed by acrylamide gel electrophoresis, Northern blotting, and hybridization to riboprobes specific for either the whole immunity region (Fig. 5) or the CI RNA (data not shown). P4 *seqA* mutants produced a higher amount of the 300- to 400-nt RNAs than did P4 *seqC*, both in the absence and in the presence (data not shown) of P4 immunity; however, none of these mutants was affected in the ability to produce the CI RNA.

DISCUSSION

Identification of *seqA* and *seqC* as targets for the P4 CI RNA immunity factor. Expression of several operons is known to be controlled by an RNA molecule rather than by a protein. In most cases analyzed so far, the RNA effector pairs to a complementary region on the transcript to be regulated (*cis*-acting element), and this interaction may be directly involved in the control of transcription elongation, RNA stability or processing, or translation efficiency (for reviews, see references 11, 27, and 34). Expression of the P4 α operon is controlled by the CI RNA coded for within the untranslated leader region of the α operon itself. The CI RNA is the *trans*-acting factor that prevents transcription of the P_{LE} distal part of the operon, thus conferring superinfection immunity (10, 11a, 12). In this work, we have presented evidence that two regions highly complementary to *seqB* within the CI RNA, *seqA* and *seqC*, are the targets of the P4 RNA immunity factor and are required in *cis* for the negative control of P4 α operon expression.

seqA was identified in a previous work (12) as a region immediately downstream of P_{LE} that exhibits complementarity to *seqB*. Since most recessive immunity-deficient mutations occurred in *seqB* and altered the complementarity to *seqA*, it was suggested that interactions between CI RNA and the nascent transcript would promote premature transcription termination of the operon (10, 12). In this work, we show that *seqA* is a key element in the P4 lysogenization mechanism since mutations in *seqA* (*seqA8* and *seqA405*) that decrease complementarity with *seqB* make P4 unable to lysogenize and efficiently shut off transcription of the α operon. However, these mutations do not allow the mutant phage to escape P4 immunity, thus suggesting that *seqA* cannot be the only *cis*-acting site interacting with the CI RNA.

The presence of a second *cis*-acting site (*seqC*) involved in the immunity control was revealed by the isolation of P4 virulent mutants that carried either double or triple mutations in the immunity region. Single mutations in *seqC* (*seqC30* and *seqC405*) reduce P4 lysogenization proficiency, whereas mutations in both *seqA* and *seqC* (P4 *seqA405 seqC405*, P4 *seqA405 cI405 seqC32*, and P4 *seqA405 cI405 seqC33*) are required for virulence. The dominant phenotype of the double mutants suggests that *seqA* and *seqC* represent the target sites for the CI RNA immunity factor. Both functional sites are necessary to efficiently establish and/or maintain immunity upon infection, whereas a single site appears to be sufficient to make a superinfecting phage susceptible to immunity already established in the host. The immunity-defective phenotype exhibited by the *seqA* and *seqC* single mutants could be accounted for by the increased persistence of the 4.1- and 1.3-kb transcripts from P_{LE} produced upon infection. These transcripts encode α and other genes necessary for P4 replication and the lytic or plasmid cycle, and a prolonged expression of such functions could prevent establishment of lysogeny and/or favor the establishment of the alternative developmental pathways.

P4 immunity requires interactions between *seqB* and *seqA* or *seqC*. Most P4 immunity-defective mutants in the *c*I gene carry a mutation in *seqB* that reduces the complementarity to both *seqA* and *seqC* (11a, 12) (Fig. 3). All of the spontaneous mutants we obtained in *seqC* affect complementarity to *seqB*, and three out of four mutants obtained by base-specific substitution in *seqA* and/or *seqC* have altered immunity. These observations indicate that complementarity between *seqB* and *seqA* or *seqC* is relevant for P4 immunity.

The most obvious target for an RNA effector is a complementary RNA molecule. Evidence that CI RNA may interact with another RNA molecule by pairing to the complementary sequence *seqA* can be found in the analysis of the anti-immunity effect. Transcription of *seqA* is required for anti-immunity, and anti-immunity may be abolished by a mutation in *seqA*, decreasing complementarity with *seqB*.

On the basis of these data, we suggest a basic model for P4 immunity. (i) The CI RNA is the *trans*-acting immunity factor. (ii) The CI RNA interacts with the RNA transcribed from P_{LE} by base pairing of *seqB* with the complementary regions *seqA* and *seqC* (Fig. 3B); intramolecular pairing of *seqB* with either *seqA* or *seqC* might occur as well. (iii) As a consequence, transcription from P_{LE} terminates prematurely and expression of P4 replication genes is prevented.

A role for *seqC* in the transcription termination mechanism is suggested by the observation that the distal part of *seqC*, *seqC″*, overlaps the ribosome-binding site and the start codon of *kil*, the first translated gene downstream of P_{LE} (11a, 12) (Fig. 3). The pairing of *seqB* with *seqC* might prevent translation of *kil* and cause premature transcription termination by a polar effect.

Mutations in *seqA* have a more drastic effect on P4 immunity and transcription termination of the α operon than do mutations in *seqC*, since no P4 lysogens could be isolated and the long transcripts from P_{LE} were abundantly expressed up to 1 h after P4 *seqA* infection. It might be suggested that *seqA-seqB* pairing may promote transcription termination, for example, by allowing the formation of alternative RNA secondary structures that favor RNA polymerase pausing and release and/or the recruitment of host transcription termination factors.

Virulent mutants of P4. Transcription from the late promoter P_{LL} is insensitive to the termination mechanism controlled by P4 immunity (10). Thus, *vir1*-like mutants, which carry mutations that make P_{LL} independent of its positive regulator (9, 20, 23a), do not provide information on the targets of the *trans*-acting immunity factor.

The second class of P4 virulent mutants we have isolated carry a mutation both in *seqA* and in *seqC*. This strongly suggests that *seqA* and *seqC* are the *cis*-acting elements required for P4 immunity sensitivity. The need for a double mutation to create this type of virulent mutant explains why the virulent mutants obtained in vivo most frequently carried a P_{LL} promoter up mutation.

A third class of virulent mutants is represented by P4 *ash29 seqC30*. The single *seqC30* mutation does not confer a virulent phenotype, so it appears that the peculiar combination of *ash29* and *seqC30* is required for P4 immunity insensitivity. An analysis of this virulent mutant might help an understanding of the molecular details of the P4 immunity process.

A compensatory mutation in *seqB* that restored complementarity with the mutant *seqA* and *seqC* (see the triple mutant P4 *seqA405 c*I*405 seqC405*) also restored premature transcription termination, therefore preventing late transcription of the whole α operon from P_{LE}. However, the compensatory mutation could not reestablish lysogenization proficiency. It may be suggested that small differences in the timing and/or efficiency of premature transcription termination may be relevant for the choice of the lysogenic condition. This could be influenced by the stringency of the *seqB* interaction with *seqA* or *seqC*. It should be noted that in these mutants, a UA pairing substitutes for the wild-type CG interaction (Fig. 3). It is possible that this weaker interaction is sufficient to substantially terminate transcription upon infection when a large amount of CI RNA is produced but may be too leaky to fully prevent α operon expression and maintain immunity. High levels of CI RNA may compensate for a weaker *seqB* interaction with *seqA* or *seqC*. In fact, P4 *seqA405 seqC405* and P4 *seqA405 c*I*405 seqC405* mutants do not grow in the presence of CI405 RNA (UA pairing) expressed by a multicopy plasmid but do form plaques with CI^+ RNA (CA mismatch); on the other hand, a P4 wild type cannot grow with either a wild-type CI (CG pairing) or CI405 (UG pairing) RNA. However, when the plasmid expressing the CI405 RNA is carried in a low copy number, both $P4^+$ and the double and triple *seq* mutants can grow (data not shown).

P4 immunity establishment versus maintenance. Both *seqA* and *seqC* single mutants are sensitive to the P4 CI immunity factor since (i) they do not grow on a P4 lysogen (in such a host, transcription of these phage mutants from P_{LE} is efficiently terminated) and (ii) in cross-streak complementation tests with either $P4^+$ or P4 *c*II, they form a turbid spot. On the other hand, both types of mutants produce a wild-type CI RNA. It is therefore somewhat paradoxical that these mutants form clear plaques, do not lysogenize (*seqA* mutants) or lysogenize at a reduced frequency (*seqC* mutants), and are unable to complement *c*I mutants. It should be considered, however, whether a delay of a reduced efficiency of the α operon transcription termination upon infection might allow the irreversible onset of the alternative lytic or plasmid pathway. Furthermore, the establishment of lysogeny may demand different (or more stringent) requirements than its maintenance. This idea is supported by other situations that greatly reduce lysogenization frequency without being incompatible with immunity maintenance, such as the anti-immunity effect due to the expression of the cloned *seqA* region or mutations in the transcription termination factor Rho of the host (8a).

Relationship to other systems. The retronphage φR73 is related to P4, and the two phages share a high degree of homology (18, 31). The putative φR73 *c*I gene sequence presents six base changes, all in *seqB*. It is noteworthy that for five of these changes, there is a complementary base substitution in both *seqA* and *seqC*, and the sixth is a C→U transition, still compatible for pairing with the G in the target sequences. φR73 is heteroimmune to P4 (27a). This supports the idea that the pairing of *seqB* to *seqA* or *seqC* is important for immunity and that immunity specificity is provided by *seqA*, *seqB*, and *seqC* sequences.

The control of P4 α operon expression exhibits striking similarities to the regulation of the phage P1 *immI* operon that codes for the P1 antirepressor (6, 24; for a review, see reference 16). The *immI* untranslated leader region is processed to produce a 77-nt RNA (C4 RNA), the *trans*-acting factor that negatively regulates the operon (5, 6). The C4 RNA bears extended complementarity with the translation initiation signals of *icd*, the first gene of the operon (5, 24). RNA-RNA interactions between these complementary sequences prevent translation of *icd*, thus preventing expression of the downstream antirepressor gene by causing Rho-dependent transcription termination (3).

The predicted secondary structures of P4 CI and P1 C4 RNAs and of the leader transcripts of the two operons are remarkably similar, and a limited sequence homology can also be detected (21). Thus, the two regulatory systems appear to be evolutionarily related.

P1 C4 RNA exhibits complementarity to both the upstream and the downstream sequences in the leader transcript. It seems, however, that the upstream sequence plays a different role in the two phages. In fact, in P1, only the downstream sequence is involved in the negative regulation of the operon, whereas the upstream sequence appears to antagonize this regulatory mechanism (5). In P4, both the upstream and the downstream sequences promote transcription termination. In P1, *c4* indirectly enables the establishment and maintenance of lysogeny by controlling the expression of an anti-immunity factor, whereas in P4, the *c*I gene encodes the immunity factor itself. It is conceivable that the different physiological roles of the two regulatory systems caused two homologous molecular structures to evolve different functions.

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